EGFR signalling upregulates expression of Microsomal Prostaglandin E Synthase-1 in cancer cells leading to enhanced tumorigenicity

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Running title: mPGES-1 modulates EGFR-mediated malignancy

Abstract

In this report we describe the contribution of prostaglandin E-2 (PG)E_2 derived from the inducible microsomal PGE-synthase type-1 (mPGES-1) to the epidermal growth factor receptor (EGFR) oncogenic drive in tumor epithelial cells and in tumor bearing mice. We investigated the mechanism controlling the mPGES-1 expression in HT-29, A431 and A549 tumor cells following EGFR stimulation, focusing on the transcriptional activity of Egr-1, a factor readily up-regulated by epidermal growth factor (EGF). The Egr-1 rise provoked the over-expression of mPGES-1 messenger and protein, and enhanced PGE_2 formation. These changes were suppressed either by silencing Egr-1, or by up-stream blockade of EGFR or ERK1/2 signals. Further, in a clonogenic assay on tumor cells, EGF induced a florid tumorigenic phenotype, which regressed when mPGES-1 was silenced or knocked down. EGF-induced mPGES-1 overexpression in epithelial cell reduced E-cadherin expression, while enhancing that of vimentin, suggesting an incipient mesenchimal phenotype. Additionally, inhibiting the EGFR in mice bearing the A431 tumor, the mPGES-1 expression and the tumor growth, exhibited a parallel decline. In conclusion, these findings provide novel evidence that a tight cooperation between the EGF/EGFR and mPGES-1 leads to a significant tumorigenic gain in epithelial cells, and provide clues for controlling the vicious association.

Key words: ProstaglandinE-2, mPGES-1, Epidermal growth factor, Egr-1, E-cadherin, Vimentin.

Introduction

Prostaglandins, mainly prostaglandin E-2 (PG)E_2, have assumed an important role in cancer biology because evidence demonstrates their involvement in cancer development as they exert a tumorigenic action. The clearest evidence has been the observation that overexpression of the inducible cyclooxygenase-2 (COX-2), and as a consequence, increased PGE_2 synthesis, was causally associated with the growth and aggressiveness of human colon cancer (Eberhart e*t al*., 1994; Wiesner *et al*., 2001). These observations were later extended to many other solid tumors (Menter *et al*., 2010).

Although PGE_2 is a well know mediator of inflammation, it is evident that PGE_2 exerts pleiotropic effects in tumors, promoting proliferation, survival, angiogenesis, migration and invasion. This multitude of PGE_2 effects has been attributed to pro-survival and proliferative signals including: PI3K/Akt (Tessner *et al*., 2004), MAPK-ERK1/2 (Pozzi *et al*., 2004), cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) (Leone *et al*., 2007), epidermal growth factor receptor (EGFR) (Pai *et al*., 2002; Buchanan *et al*., 2003; Donnini *et al*., 2007) and activation of β-catenin/TCF in colorectal cancer cells (Castellone *et al*., 2005).

Recent investigations have focused on PGE_2 synthases (mPGES-1, mPGES-2 and cPGES), specifically on mPGES-1, the only inducible enzyme among those so far identified in cells. Whereas cPGEs and mPGEs-2 are constitutively expressed at relatively low levels, mPGES-1 is highly inducible (Samuelsson *et al*., 2007). All enzymes, being downstream of COX-2, act selectively on PGE_2 synthesis, thus circumventing the issue of blocking the formation of protective prostaglandins associated with COX-2 inhibitors.

mPGES-1 upregulation has been detected in many epithelial tumors (Yoshimatsu *et al*., 2001a; Yoshimatsu et al., 2001b; Golijanin *et al*., 2004¸Choe *et al*., 2005), and its silencing has been reported to reduce pre-neoplastic lesions (Nakanishi *et al*., 2008). mPGES-1 tumorigenic potential has also been demonstrated in cell cultures, exemplified by HEK293 cells, in which co-

transfection of COX-2 and mPGES-1 induced an increase in proliferation in vitro, and in vivo, when these engineered cells were inoculated in nude mice, formed large and vascularized tumors (Kamei *et al*., 2003; Kamei *et al*., 2009). Despite the evidence linking mPGES-1 and tumorigenesis, most studies investigating the inducible nature of the enzyme in cultured cells have been performed using a variety of inflammatory stimuli (LPS, IL-β, TNFα and others) (Naraba *et al*., 2002; Subbaramaiah *et al*., 2004; Cheng *et al*., 2004), while little is known about the behavior of the enzyme in tumor cells challenged with oncogenic stimuli.

Here, we investigated mPGES-1 regulation in cultured epithelial tumor cells exposed to epidermal growth factor (EGF), an oncoprotein which, through its receptor EGFR, is responsible for tumorigenesis in a wide array of solid tumors (Ciardiello and Tortora, 2008). We used three cell lines, representative of colon (HT-29), epidermoid (A431), and lung (A549) tumors, examining the mPGES-1 expression following EGFR activation by EGF. We also studied in detail the expression of transcription factors (e.g. Egr-1, NFkB) relevant to the enzyme, as well as the signalling pathway downstream of EGFR activation (ERK1/2). The tumorigenic profile of epithelial cells was analyzed by the clonogenic assay, and by evaluating the potential development of a mesenchimal transition phenotype through the assessment of e-cadherin and vimentin expression in either wild type or mPGES-1 knockdown tumor cells. We also investigated the effect of inhibiting the EGFR in tumor bearing mice on mPGES-1 expression and tumor growth, finding a parallel decline of both. The results of this study provide further evidences for the pro-tumorigenic role of PGE_2 in epithelial cells of diverse lineage as we demonstrate the existence of a tight link between the EGF/EGFR, Egr-1, and mPGES-1 molecular pathway and tumor progression.

Results

EGFR activation up-regulates mPGES-1 expression

The EGFR ligand, EGF (25 ng/ml) up-regulated mPGES-1 expression both at protein (18 h, Fig. 1A and B) and messenger level (6 h, Fig. 1C) in all cell lines, colon HT-29, epidermoid A431, and lung A549 carcinoma cells. The enzyme up-regulation was accompanied by increased PGE_2 secretion, ranging from 2.3 to 2.7-fold over control, depending on the cell line (Fig. 1D). On the contrary, neither mPGES-2 nor cPGES expression were significantly modified by EGF treatment (Fig. 1A). EGF also induced COX-2 expression, although in a manner which was variable in time and extent, depending of the cell line (Fig. 1A, see legend).

To further explore the EGFR-induced up-regulation of mPGES-1, we studied mPGES-1 promoter-driven transcription in the above cell lines, after transfection with plasmids bearing fragments of the mPGES-1 promoter differing in nucleotide length (from-1100 to +30). EGF consistently augmented mPGES-1 transcription, expressed either as fold induction relative to the empty plasmid, or absolute values of relative luciferase units (RLU) (Fig. 2A and table 1, respectively). The variable extent of mPGES-1 induction observed in the cell lines used was dependent on cell phenotype and on the length of the mPGES-1 promoter (table 1). The shorter plasmid (from -154 to +30), containing GC-rich elements, was sufficient to drive the mPGES-1 transcription (Fig. 2A). This suggest that Egr-1, an inducible zinc finger protein that recognizes the GC-rich consensus DNA sequence, is involved in EGF-mediated mPGES-1 expression.

mPGES-1 up-regulation requires ERK1/2 and Egr-1 activation downstream to EGFR

We studied the expression of Egr-1 in tumor cells exposed to EGF. Because EGF /EGFR signalling is known to promote solid tumor growth via activation of the MAPK/ERK1/2 pathway (Dasari and Messersmith, 2010) we evaluated ERK1/2 involvement in the expression of mPGES-1.

Indeed, EGF (25 ng/ml) induced Egr-1 expression and ERK1/2 phosphorylation in the three cell lines analysed in a time-dependent manner, both events peaking between 15 and 45 min (Fig. 2B).

Next, we studied the EGF-induced activation of Egr-1 analyzing both its translocation from the cytosol to the nucleus (Western blot and immunohistochemistry), and the induction of its transcriptional activity. Egr-1 translocation was evidenced by the time-related enrichment of protein or fluorescence into the nucleus of HT-29 cells (Fig. 2C and D). Similarly, we observed an increase, in all cell lines, of luciferase activity of Egr-1-Pro36LUC, a construct containing two Egr-1 binding sites inserted upstream of a prolactin minimal promoter (24) (Fig. 2E), demonstrating that EGF induces both Egr-1 expression and activity.

A number of experiments firmly established the involvement of EGFR-MAPK pathway in Egr-1 and mPGES-1 expression. Thus, blockers of either EGFR or ERK1/2 (AG1478, and U0126, respectively) suppressed the EGF-induced up-regulation of both Egr-1 and mPGES-1 in all cell lines examined (Fig. 2F).

The involvement of Egr-1 in the inducing mPGES-1 in tumor cells, was further documented by experiments in which Egr-1 expression was knocked down. In fact, silencing Egr-1 in epithelial tumor cells, abolished its overexpression provoked by EGF (Fig 3A), concomitantly reducing the mPGES-1 induction (Fig 3A). Conversely, in experiments in which tumor cells were transfected with the Egr-1 expression plasmid pLNCX-NGFI-A (Clone Egr-$1^{+/+}$) we found that higher levels of Egr-1 expression (clone Egr-1+/+) corresponded to higher mPGES-1 protein levels (Fig. 3B, $p<0.001$). Further, in tumor cells silenced for Egr-1, EGF failed to induce mPGES-1 transcription (Fig. 3C). Additional evidence for the Egr-1 role was obtained by performing the ChIP assay in HT-29 cells treated with EGF for 45 min. In these conditions Egr-1 was found to be specifically bound to the mPGES-1 promoter only in cells stimulated by the growth factor, whereas its binding to control cells was negligible (Fig 3D). Thus, EGF appears to promote the recruitment of Egr-1 toward the mPGES-1 promoter.

All together these data demonstrate the requirement for ERK1/2/Egr-1 pathways downstream of EGFR for induction of mPGES-1 expression.

We also explored whether the mPGES-1 gene was regulated by NFkB, a transcription factor known to convey inflammatory and oncogenic stimuli to a wide variety of genes (Lin and Karin, 2007). Pre-treatment of HT-29 cells with IKK, an inhibitor of NFkB that leads to inhibition of NFkB activity, suppressed the EGF-induced mPGES-1 expression, suggesting the involvement of this transcription factor in EGF-dependent induction of mPGES-1 gene in tumor cells (data not shown).

Silencing mPGES-1 expression reduces epithelial cell tumorigenicity

The role of mPGES-1 expression on the cell tumorigenic potential following EGFR activation was assessed by the clonogenic assay. While EGF induced colony formation in both HT-29 and in A549 cells, mPGES-1 gene knockdown in either cell types, reduced by 3 to 5 fold the EGFR response (Fig. 4A).

This suggests that mPGES-1/ PGE_2 signaling is implicated in the malignancy induced by EGF. Because the regulation of E-cadherin and vimentin are well established biomarkers of enhanced malignancy, signaling the initiation of epithelial-mesenchymal transition (EMT) process (Thiery *et al*., 2009), we investigated their expression in A549 cell types. Indeed, in A549 cells wild type, or transfected with non target shRNA, EGF decreased E-cadherin, while increasing vimentin expression (Fig. 4B). Conversely, in A549 knocked down for mPGES-1, EGF failed to regulate both proteins-mediated adhesion/movement. Thus mPGES-1 appears to control the EGF/EGFR oncogenic drive by governing the development of the EMT program (Fig. 4B).

Tumor growth decline, caused by EGFR blockade, is associated to the downregulaton of mPGES-1 expression

The functional association between EGFR and mPGES-1 signaling was further investigated in mice bearing human A431 xenograft, which expresses high EGFR levels (Johns *et al*., 2002). A431 cells (10^7 cells) were inoculated in nude mice. Treatment with vehicle control (0.5% methylcellulose) or the EGFR inhibitor AG1478, (400 μg/mice, 10 days) started 4 days after cells implantation, a time at which tumors were measurable (3 mm diameter). Tumor size in controls increased steadily, reaching an average volume of 400 mm^3, 7-fold higher at day 10, relative to day 4. AG1478 administration reduced tumor growth by 50% starting from day 8 ($P < 0.01$), relative to the vehicle- group (Fig. 5A). During the course of treatment neither body weight loss nor signs of toxicity were observed. In AG1478 treated tumors, mPGES-1 expression, analyzed by Western blot and immunohistochemistry, was significantly reduced relative to control specimens (Fig. 5B and C, $p<0.05$). Consistently, mPGES-1 expression in tumor samples correlated with tumor volume (Fig. 5B). Indeed, treatment of human A431 xenograft with selective inhibitors of mPGES-1 activity, was found to reduce both growth and receptor phosphorylation of tumors (manuscript in preparation).

Discussion

The keen interest in prostaglandin biosynthesis stems from the recognition that PGE_2 exerts an important role in the initiation and progression of several epithelial tumors (Menter *et al*., 2010). In this study we focused on microsomal prostaglandin synthase (mPGES-1) examining its inducible behavior in cultured epithelial tumor cells (HT-29, colon, A431, squamous cell, and A549, lung adenocarcinoma) following stimulation by EGF. Although a number of reports have described the relationship between mPGES-1 overexpression and tumor growth, the evidence that the enhanced malignancy is linked to a transduction loop between the prostanoid and the EGF system, is fragmentary (Wang and Dubois, 2010, Hanaka *et al*., 2009; Nah *et al*., 2010; Lu *et al*., 2011). Our aim was to demonstrate that the mPGES-1/PGE2 and the EGF axis provides a cohesive program for malignancy in epithelium. Here, we show that EGFR stimulation induces mPGES-1 up-regulation and increases PGE_2 production, through the specific activation of gene transcription pathway, i.e.Egr-1, in epithelial tumor cells of diverse lineage (see below). In addition, we show that epithelial cells expressing mPGES-1 evolve toward a distinct tumor phenotype, and produce a fast-growing tumor mass when inoculated in nude mice.

The EGF induced mPGES-1 over-expression, preceded by the rise of its encoding mRNA, was similar in the three cell lines examined. In the promoter region of the mPGES-1 gene, several binding sites for transcription factors have been identified, including GC boxes for Egr-1, NFkB, AP-1 and c/EBP response elements (Diaz-Munoz *et al*., 2010). The detailed analysis of the mPGES-1 promoter (from-1100 to +30), performed by transfecting tumor cells with mPGES-1 promoter constructs of different length, revealed a consistent enhancement of the EGF-driven mPGES-1 transcription, enabling also to identify the minimal sequence, containing solely the Egr-1 binding sequence, capable of eliciting a response. The ChIP assay corroborated these observations by showing the Egr-1 specific binding within the mPGES-1 promoter. Thus, these findings delineate the role of Egr-1 in the EGFR-induced expression of the mPGES-1 gene in epithelial

tumor cells. The interplay between mPGES-1 and Egr-1 expression was clearly demonstrated by experiments involving either Egr-1 silencing through siRNA, or forcing its expression through transfection with the pLNCX-NGFI-A Egr-1 plasmid, resulting in abrogation of mPGES-1 promoter activity and expression or mPGES-1 over-expression, respectively. Although, these data clearly point to Egr-1 as an important transcription factor of mPGES-1 gene expression following EGF/EGFR stimulation, one ought to consider the vast redundancy of signals present in neoplastic epithelial cells. Among the wide repertoire of pro-inflammatory stimuli known to induce mPGES-1 gene (PMA, IL-1β, TNFα or LPS), recently some of us described the involvement of NFkB in both Egr-1 and mPGES-1 expression in macrophages exposed to LPS (Diaz-Munoz *et al*., 2010). Indeed, in HT-29 tumor cells, inhibition of NF-kB activity by the use of an IKK inhibitor prevented the EGF-induced mPGES-1 over-expression, suggesting that also in tumor cells the mPGES-1 transcription could be controlled by several transcription factors, whose action might be exerted in cell and in a context-dependent manner (Naraba *et al*., 2002; Cheng *et al*., 2004; Diaz-Munoz *et al*., 2010 ; Deckmann *et al*., 2010). Inhibition of NF-kB activity precludes from distinguishing from its transcriptional effect on mPGES-1 by direct binding of mPGES-1 promoter or indirectly, by altering Egr-1 expression (Diaz-Munoz *et al*., 2010).

The connection between EGF/EGFR and mPGES-1 in promoting tumorigenicty in epithelial cells, so far largely surmised from work on non-tuor cells (e.i. synoviocytes) (Nah *et al*., 2010), is here clearly demonstrated by two lines of evidence. First, the clonogenic assay shows that abrogation of the mPGES-1 gene in tumor epithelial cells (HT-29), markedly reduces the EGF tumorigenic potential. Second, the in vivo demonstration that blockade of EGFR by AG1478 (a TK receptor inhibitor) in a xenograft model of epithelial tumor (A431) produces a decrease of tumor mPGES-1 expression while reducing tumor growth. To be noted that this tumor line features the highest inducible mPGES-1 and PGE_2 levels among the cells examined (see Fig 1) and a very robust EGF/EGFR system (Thoren and Jakobsson, 2000).

The PGE_2 leveraging effect on EGF tumorigenic action has been explained according to various mechanisms, among which, the transactivation of EGFR has been the most studied (Pai *et al*., 2002; Buchanan *et al*., 2003; Donnini *et al*., 2007). However, the reported mechanisms, involving the regulation of E-cadherin expression by PGE_2, might contribute to enhance tumorigenesis in solid cancer (Mann *et al*., 2006 ; Dohadwala *et al*., 2006). For example, PGE_2 has been shown to suppress E-cadherin expression, through the transcriptional repression of Snail and ZEB genes (Dohadwala *et al*., 2006). Furthermore, the EGF-induced Snail expression leads to PGE_2 increase in colon tumor cells by inhibiting PGDH, the main degrading enzyme for prostanoids in tumors (Mann *et al*., 2006). It is of interest that Egr-1 has been reported to induce Snail by binding to its promoter, thus acting as a trigger for the epithelial mesenchymal transition (Grotegut *et al*., 2006). Indeed, the marked changes of E-cadherin and vimentin expression, noted here in A549 wild type but not in mPGES-1 knock down, provide evidence for the relevance of mPGES-1 in the incipient epithelial to mesenchymal transition, a process which confers enhanced tumorigenic properties to epithelial cells, in terms of invasion and ability to form metastasis. Additionally, the mPGES-1/PGE_2 biosynthetic pathway might contribute to cancer progression through other cytokine/growth factors, as exemplified by the reported synergism between PGE_2 and FGF-2/FGFR1 system in sustaining tumor vascularity (Finetti *et al*., 2009).

The number of mechanisms in which PGE_2 and consequently mPGES-1, is involved in boosting the EGF-driven tumorigenicity in epithelial cells, signals its key role in rewiring cells toward a tumor phenotype. Conceivably, strategies aimed to interfere with mPGES-1 signaling might be a valuable addition to the armamentarium for controlling epithelial tumor progression.

Materials and methods

Reagents

Reagents were as follows: AG1478, IKK inhibitor VII and U0126 (Calbiochem, Darmstadt, Germany), anti-β-actin, anti-vimentin, (Sigma, Milan, Italy); anti-mPGES-1, anti-mPGES-2, anti-cPGES and anti-COX-2 antobodies (Cayman Chemical, Vincibiochem, Florence, Italy); EGF (RELIAtech, Wolfenbuttel, Germany), anti phospho-p44/42 MAPK, anti-EGFR (Cell Signalling, Pero, Italy); anti Egr-1 (Santa Cruz, Heidelberg, Germany); anti-E Cadherin antibodies (DAKO, Milan, Italy). Where not indicated, reagents were from Sigma.

Cell Lines

HT-29, human colorectal adenocarcinoma cells, A431 human epidermoid carcinoma and A549 human lung carcinoma cells were obtained from ATCC and cultured as recommended. A549 wild type (WT), mPGES-1 knockdown (Kd) and non-target shRNA cells obtained and cultured as described (Hanaka *et al*., 2009).

Western blotting

4 x 10^5 cells were plated in 60 mm dishes, serum deprived (0.1% FCS, 24 h), then exposed to EGF in the presence or absence of MAPK, EGFR or NFκB inhibitors. To assess the translocation of Egr-1 from cytosol to nucleus, after treatment with EGF for the indicated times, cells were scraped/trypsinized, and homogenized on ice in a lysis buffer, containing 0.1 mM EGTA, 0.1 mM EDTA,10 mM Hepes, 10 mM KCl, protease and phosphatase inhibitors. After incubation on ice for 15 min, Nonidet-P-40 was added to cell lysates which were centrifuged (10.000 rpm, 30 sec). The supernatant contains the cytosolic fraction while the pellet was solubilized in lysis buffer containing 1 mM EGTA, 1 mM EDTA, 20 mM Hepes, 10 mM NaCl, 1%protease and phosphatase inhibitors, followed by incubation on ice for 10 min and centrifugation (14.000 rpm, 5 min). The supernatant

contains the nuclear fraction. An equal amount of proteins were loaded on SDS PAGE gel and then transferred to a nitrocellulose membrane. Western blotting was performed as described (Donnini *et al*., 2007). Images were digitalized with CHEMI DOC Quantity One programme, blots were analysed in triplicate by densitometry using NIH Image 1.60B5 software, and the arbitrary densitometric units (ADU) were normalized to ADU for β-actin.

PGE_2 immuno-assays

PGE_2 was measured by an EIA kit (Prostaglandin E_2 EIA kit-Monoclonal, Cayman Chemical). Cells were exposed to EGF (25 ng/ml, 24 h) and treated with 10 μM arachidonic acid. Cell culture supernatants were assayed directly at a final dilution of 1:10 to 1:500. PGE_2 concentration was expressed as [pg/ml], normalized to total protein concentration.

Real-Time PCR

Total RNA was obtained using RNA mini kit (Qiagen, Inc., Milan, Italy). RNA (0.5 μg) was reverse transcribed using a RT-PCR kit (Applied Biosystems, Foster City, USA). mPGES-1 mRNA detection was measured using the optimized TaqMan assay-on-demand (Applied Biosystems) and the expression of the mRNAs in each sample was calculated by referring to an external reference curve generated with universal human reference RNA (Stratagene, La Jolla, USA). The results were expressed as fold increase.

Luciferase activity

Cells were transiently transfected with luciferase constructs containing different deletions of the murine promoter of mPGES-1 cloned in the pxP2-LUC plasmid: mPGES-1-1100 (-1100 to +30) mPGES-1-895 (-895 to +30), mPGES-1-483 (-483 to +30) and mPGES-1-154 (-154 to + 30), or

with a construct containing two Egr-1 consensus binding sites insert upstream of a prolactin minimal promoter (Egr-1-Pro36LUC) (Diaz-Munoz *et al*., 2010; Crosby *et al*., 1991).

Transfections were performed using Effectene Transfection Reagents (Qiagen, Milan, Italy) according to manufacturer's instructions. Transfection efficiency was assessed by co-transfection with a plasmid harbouring the Renilla luciferase gene under control of a constitutive promoter (Promega, Madison, USA). 24 h following transfection, cells were starved for 24 h and stimulated for 18 h (mPGES-1 LUC) or 45 min (Egr-1-Pro36LUC) with EGF (25 ng/ml) and then lysed. Luciferase reported assays were performed using Steady Glo and dual luciferase reporter assay reagents (Promega), and activity was measured using a Tecan Infinite 200Pro Luciferase activity was normalized according to the protein expression for each condition.

Transfection

For siRNA transfection: the siRNAs sequences (human mPGES-1: 5'-CGGGCTAAGAATGCAGACTTT-3', Egr-1: 5'-CCCGTCGGTGGCCACCACGTA-3') were from Qiagen. The day before transfection, cells were trypsinized and 3 x 105 cells were seeded in 6-well plates. Transient transfection of siRNA was carried out using HT-29 transfection reagent (Altogen, Las Vegas, Nevada, USA) according to the manufacturer instructions. Cells were assayed 48 h after transfection.

For DNA transfection: transient transfection of Egr-1 expression plasmid pLNCX-NGFI-A (Clone Egr-1+/+) generously provided by Dr. A. M. Pérez-Castillo (Instituto de Investigaciones Biomédicas,Madrid, Spain) (Pignatelli *et al*., 1999), was carried out using Effectene transfection reagent (Qiagen, Italy) according to the manufactures instructions. Cells were assayed 48 h after transfection.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay (ChIP) assays were performed as described (Díaz-Muñoz et al. 2010). HT-29 cells (6×10^6) were maintained in RPMI with 0.5% FCS for 24 h prior stimulation with EGF for 45 min. Cells were then fixed with 1% formaldehyde for 5 min at 37 °C and lysed in ice-cold lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40 and protease inhibitors) for 10 min at 4 °C. Nuclei pellet was suspended in nuclear lysis buffer (50 mM Tris–HCl pH 8, 10 mM EDTA, 1% SDS and protease inhibitors) and incubated on ice for 10 min. DNA was shared by sonication and lysates were cleared by centrifugation and diluted in ChIP dilution buffer (50 mM Tris–HCl pH 8, EDTA 5 mM, NaCl 200 mM, and 0.5% NonidetP-40). Lysates were precleared with salmon sperm/protein A-agarose. A sample of "input DNA" (positive control (+)) was collected at this point. Protein–DNA complexes were immunoprecipitated overnight at 4 °C with 2 μg of the anti-Egr-1 or non-immune rabbit serum as a control (negative control (-)). Antibody–protein–DNA complexes were then captured using salmon sperm DNA/protein A agarose for 30 min followed by washes with wash buffer (20 mM Tris–HCl pH 8, 2 mM EDTA, 0.1% SDS, 1% NP-40, and 500 mM NaCl) and TE buffer (20 mM Tris–HCl, and 2 mM EDTA). The protein/DNA complexes were eluted using extraction buffer (20 mM Tris–HCl, 2 mM EDTA, and 2% SDS) and disrupted by heating at 65 °C overnight followed by proteinase K treatment for 2 h at 45 °C. DNA was extracted with a DNA Purification system (Promega). PCR was conducted using promoter specific primers (Applied Biosystem,): mPGES-1, sense 5′-TCC GGC AAC TGC TTG TCT TTC TCT-3′ and antisense 5′- TGT GAT CAG CTC GAC AGA GGA GCA-3′. PCR products obtained after 35 cycles were separated on 2% agarose gels.

Immunofluorescence analysis

HT-29 cells ($3x10^4$ cells/well on glass cover-slips placed into 24 multiwell plates) were serum starved and treated with EGF for 45 min. Cells were fixed in paraformaldehide for 5 min and then

washed in PBS with Ca^{2+} and Mg^{2+}. Cells were then permeabilized in 0.25% Tween-20 in PBS for 10 min. After the blocking of unspecific bindings in 3% bovine serum albumin (BSA) for 30 min the cells were incubated overnight at 4°C with a polyclonal antibody against Egr-1 (Santa Cruz) diluted 1:40 in PBS/0.5% BSA. Samples were then incubated with secondary antibody TRITC conjugated (Sigma) and assessed by fluorescence microscope (Eclipse TE300, Nikon) at 40X magnification and images taken by a digital camera.

Clonogenic assay

For clonogenic assay, HT-29 cells, after silencing for mPGES-1, and A549 cells, WT, non-target shRNA, or Kd were incubated with EGF (25 ng/ml) for 18 h. Following EGF treatment, cells were plated in 60 mm culture dishes (HT-29 at a density of 2000 cells/dish, A549, 150 cells per dish) in medium containing 10% FCS, and then kept in a humidified incubator at 37°C and 5% CO_2 for 3 or 2 weeks, respectively. Colonies (>75cells) with 50% plate efficiency were fixed and stained with 0.05% crystal violet (Sigma, Italy) in 10% ethanol and counted.

In vivo tumor xenograft

Experiments have been performed in accordance with the EC guidelines and National Ethical Committee. Immunodeficient mice (5 week-old female athymic nude mice, Harlan, USA) were s.c. inoculated in the right flank with 10^7 A431 cells in 50 µl/PBS. After 4 days, when tumors reached a 70-100 mm3 volume, animals were randomly assigned to 2 different experimental protocols. At this time i.p. AG1478 treatment (400µg/mouse, daily, 8 mice), or vehicle (0.05% methylcellulose, 8 mice) started. Mice were treated with 200 µl volume i.p., for 10 consecutive days. Data are reported as tumor volume (mm3). Animals were observed daily for signs of cytotoxicity, at day 10 were sacrificed by CO2 asphyxiation, and tumors collected and split in two parts. One part was

immediately frozen in liquid nitrogen for Western blotting as described (1), the other part was embedded in Tissue-Tek O.C.T. (Sakura, San Marcos, USA), for histology. Seven thick cryostat sections from tissue samples were stained with hematoxylin and eosin, and adjacent sections were used for immunohistochemical staining with the anti-mPGES-1 (Cayman). Cryostat sections were first fixed in acetone 20°C and incubated for 10 min in 3% H2O2, washed in TBS, then incubated in a blocking reagent (KIT Immunoperoxidase Secondary Detection System, Chemicon). Anti-m-PGES-1 diluted 1:100 (5 µg/ml) in TBS and 0.05% BSA was applied for 1 h at room temperature. Sections were then washed (TBS) and incubated for 10 min in the appropriate species-specific biotinylated secondary antibodies (goat anti-rabbit IgG, KIT Immunoperoxidase Secondary Detection System, Chemicon). After washing, the sections were incubated for 10 min in streptavidin-conjugated HRP and exposed to 3,3-diaminobenzidine tetrahydrocloride for 8 min to produce a brown reaction product. Sections were then counterstained in hematoxylin and mounted in Aquatex (Merck, Rahway, NJ, USA). Western blotting analysis for mPGES-1 was performed as reported above (see Western blotting analysis).

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed using Student's t test, analysis of variance (ANOVA) or Student-Newman-Keuls test for multiple comparisons. $P<0.05$ was considered statistically significant.

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Figure legends

Figure 1. EGF promotes mPGES-1 expression and activity. (A) Analysis of mPGES-1, mPGEs-2, cPGEs and COX-2 in three cancer cell lines (HT-29, A431 and A549) in response to EGF (25 ng/ml, 18 h for mPGES-1, mPGES-2 and cPGES, and 4 h for COX-2 in HT-29 and A431, and 8 h in A549). (B) Quantification of mPGES-1 expression in HT-29, A431 and A549. Data are reported as Arbitrary Density Unit (ADU) and represents the ratio between mPGES-1/Actin expression of three independent blots (***P<0.01). (B) Quantitative PCR for mPGES-1 mRNA expression in cells exposed to EGF (25 ng/ml) or fresh medium (Control, 6 h). mPGES-1 mRNA expression reported as fold increase compared to basal levels (n=3). (***P<0.001; **P<0.05). (C) EIA immunoassay for PGE_2 production, expressed as [pg/ml] (n=3), in cells treated with EGF (black bar, 25 ng/ml) or fresh medium (white bar, Control) in presence of arachidonic acid (10 μM, 24 h) (***P<0.001).

Figure 2. EGF promotes mPGES-1 transcriptional activity. (A) mPGES-1 transcription activity monitored through a luciferase assay with different mPGES-1 promoter constructs (described in material and methods) in cells exposed to EGF (25 ng/ml) or fresh medium. Data are shown as fold increase (EGF mediated RLU/basal RLU in absence of any stimulus). Results are representative of at least three independent assays. (B) ERK1/2 activity and Egr-1 expression in tumor cells exposed to EGF (25 ng/ml). Gel representative of three with similar results. (C) Western blotting analysis of Egr-1 expression in cytosol and nucleus in HT-29 exposed to EGF (25 ng/ml) for the indicated times. (D) Immunofluorescence analysis of Egr-1 expression (red) in HT-29 exposed for 45 min to EGF (25 ng/ml). Cell nuclei are revealed by DAPI staining (blue). White arrows in panels "EGF 45" indicate the increased fluorescence in nucleus. Scale bars indicate 100 μm. Images taken at 60X. (E) Transcriptional activity of Egr-1 monitored through a luciferase construct in cells exposed to EGF (25 ng/ml) or fresh medium (45 min), *P<0.01. (F) ERK1/2 activity (15 min), Egr-1 (45 min) and mPGES-1 expression (18 h) in HT-29 exposed to EGF (25 ng/ml) in HT-29 cells

pretreated (30 min) with or without AG1478 (10 μM), or U0126 (10 μM). Gel representative of three with similar results.

Figure 3. EGF-induced mPGES-1 expression is mediated by ERK-1/2 activity and Egr-1. (A) Western blot analysis of mPGES-1 in HT-29 transiently transfected with non targeting siRNA (siCont) or siRNA for Egr-1 (siEgr-1), and then exposed to EGF (25 ng/ml) or fresh medium for 18 h. (B) HT-29, A431 and A549 cells were transiently transfected with the Egr-1 expression plasmid pLNCX-NGFI-A (Clone Egr-$1^{+/+}$) and analyzed by western blot for expression of Egr-1 and mPGES-1 versus Actin. Gel representative of at least three with similar results. (C) mPGES-1 transcription activity in A549 cells silenced for Egr-1 (si-Egr-1) or with a random nucleotide (si-Cont) and then exposed to EGF (25 ng/ml) or fresh medium . Data are expressed as luciferase activity in RLUs±SD. Results are representative of at least three independent assays. (D) Analysis of the specific binding of Egr-1 to mPGES-1 promoter region in Ht-29 cells by ChIP assays. Egr-1 transcription factor was immunoprecipitated from cells stimulated with EGF (25 ng/ml) for 45 min. Immunoprecipitated DNA was amplified with specifics primers for the mPGES-1 proximal promoter region. As a positive control, PCR was performed on chromatin fragments isolated before immunoprecipitation (+). Immunoprecipitation with a normal rabbit serum was carried out in parallel as negative control (-).Shown is a representative experiment of the three experiment performed.

Figure 4. Knockdown of mPGES-1 reduces clonogenicity of tumor cells. (A) Colony formation capability of HT-29 cells, (si-Cont, and si-mPGES-1 clones), and A549 cells, (non-target shRNA, and mPGES-1 knockdown clones) in response to EGF . Colonies (>75 cells) with 50% efficiency were counted. Results are expressed as the increase (Δ) number of colonies in HT-29 cells and A549 in response to EGF over control (*$P<0.01$). The absolute number of colonies ± SD of three experiments was: HT-29, si-Cont, Ctr: 15 ± 2, EGF: 70 ± 10; si-mPGES-1, Ctr: 20 ± 7; EGF: 28 ± 6; A549, non-target shRNA, Ctr: 16 ± 5; EGF: 53 ± 3; mPGES-1 knockdown clones, Ctr: 25 ± 4;

EGF: 28 ± 7. (B) Western blot analysis for E-cadherin and vimentin expression in A549 wild type (WT), non-target shRNA and mPGES-1 knockdown (Kd) cells exposed to EGF (25 ng/ml) for 48 h.

Figure 5. AG1478 inhibits A431 tumor growth in xenograft nude mice. (A) Athymic mice (8 in each group) were inoculated with A431 cells and treated with AG1478 (400 μg/mice) or vehicle (Ctr, 0.05% MTC) (*P<0.005, **P<0.01). (B) Western blot analysis of mPGES-1 in xenograft tumor tissues (50 μg total proteins/lane). Quantification of mPGES-1 expression in tumors is reported as Arbitrary Density Unit (ADU) and represented the ratio between mPGES-1 and Actin expression. For each tumor sample analyzed for immune-histochemistry, it is shown their volume as mm^3. (C) Representative images of histopathological analysis of mPGES-1 (brown) in tumor sections from control (top) or AG1478 (bottom) treated mice. Scale bars indicate 100 μm. Images taken at 40X.

Figure 1

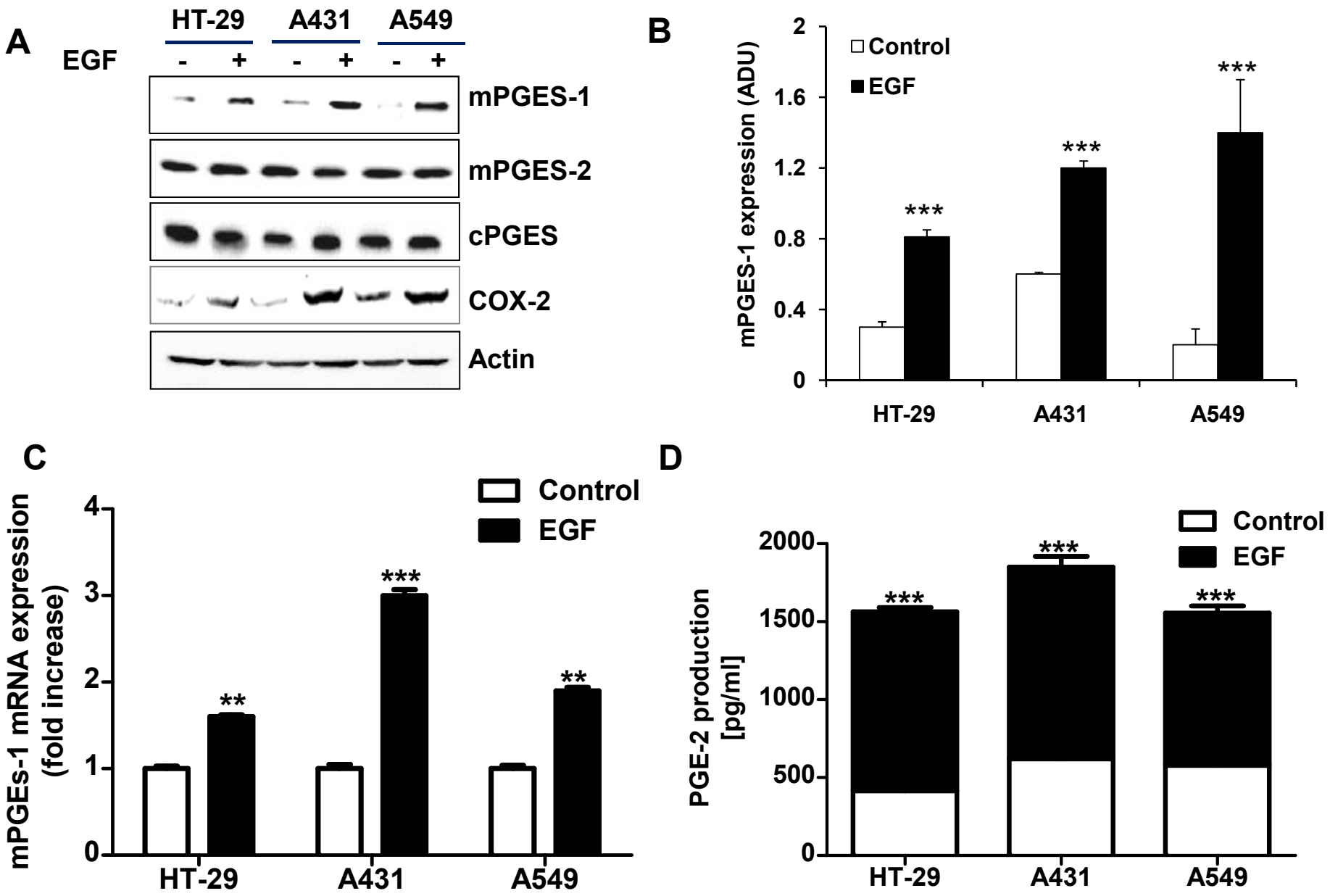

A
HT-29
A431
A549
EGF
- + - + - +
mPGES-1
mPGES-2
cPGES
COX-2
Actin
B
Control
EGF
mPGES-1 expression (ADU)
2
1.6
1.2
0.8
0.4
0
***
***
***
HT-29
A431
A549
C
Control
EGF
mPGEs-1 mRNA expression (fold increase)
4
3
2
1
0
**
***
**
HT-29
A431
A549
D
Control
EGF
PGE-2 production [pg/ml]
2000
1500
1000
500
0
***
***
***
HT-29
A431
A549

Figure 2

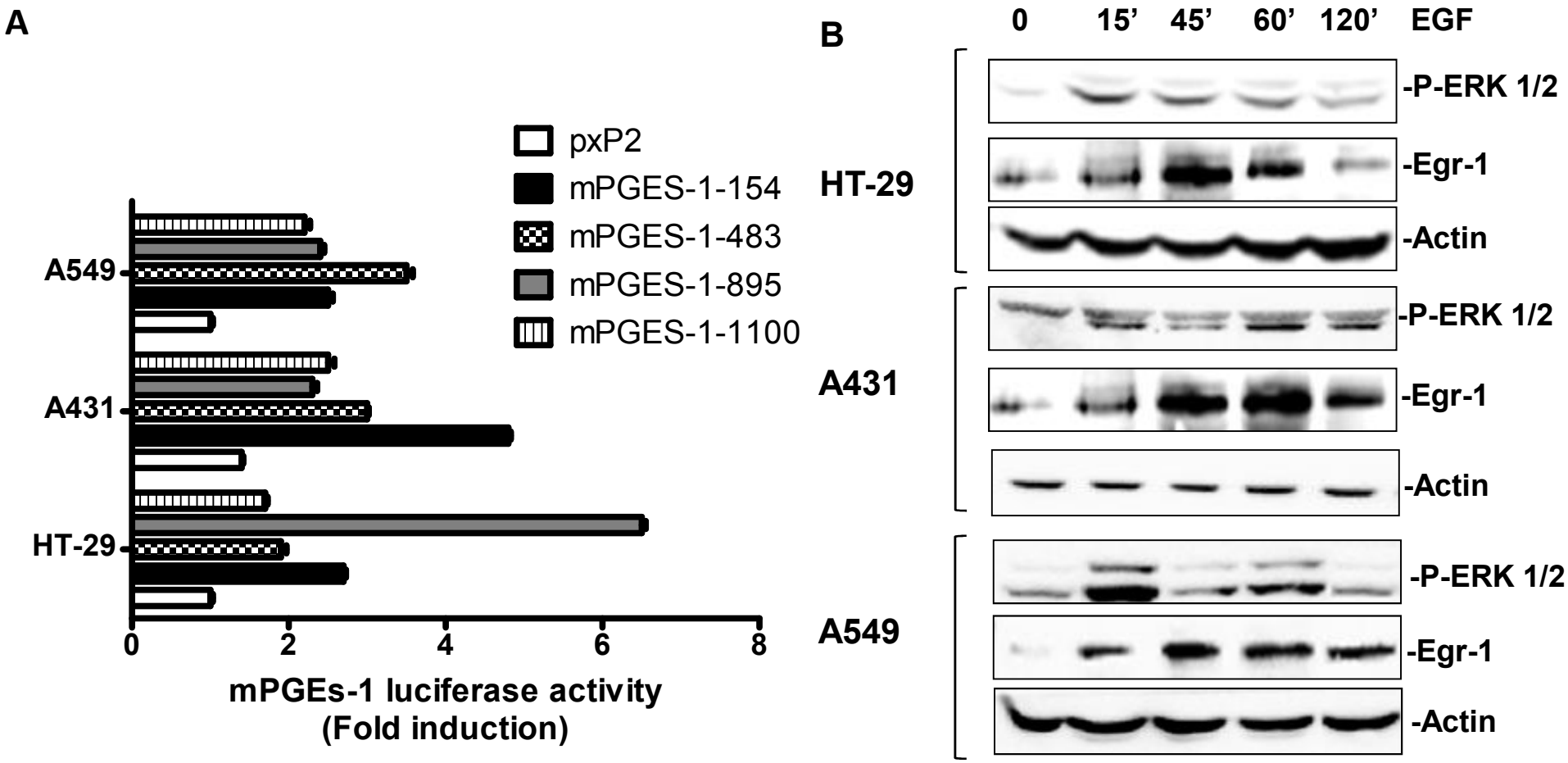
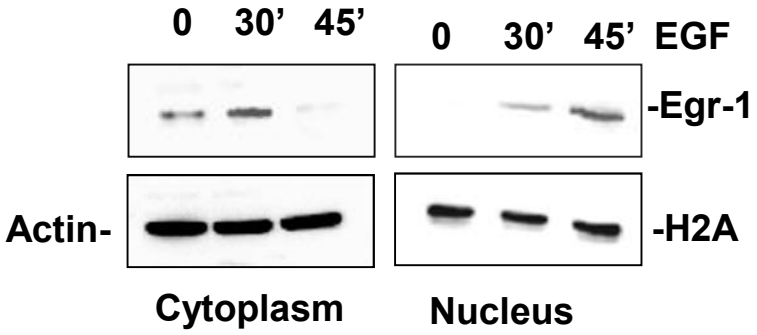

A
pxP2
mPGES-1-154
mPGES-1-483
mPGES-1-895
mPGES-1-1100
A549
A431
HT-29
0
2
4
6
8
mPGEs-1 luciferase activity
(Fold induction)
B
0
15'
45'
60'
120'
EGF
HT-29
-P-ERK 1/2
-Egr-1
-Actin
A431
-P-ERK 1/2
-Egr-1
-Actin
A549
-P-ERK 1/2
-Egr-1
-Actin

Figure 2

C



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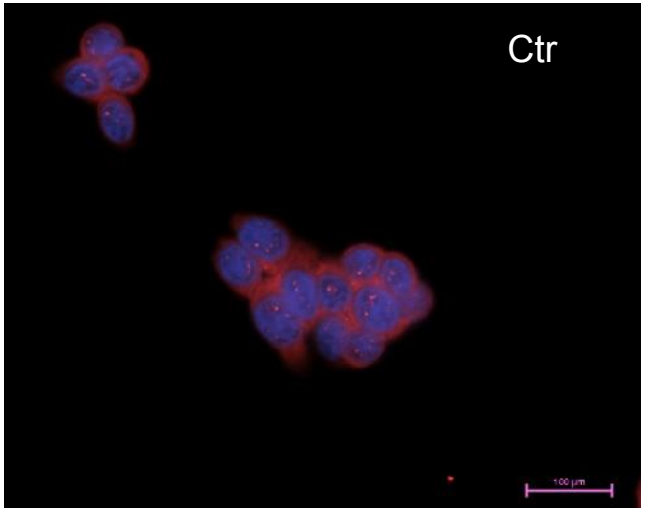


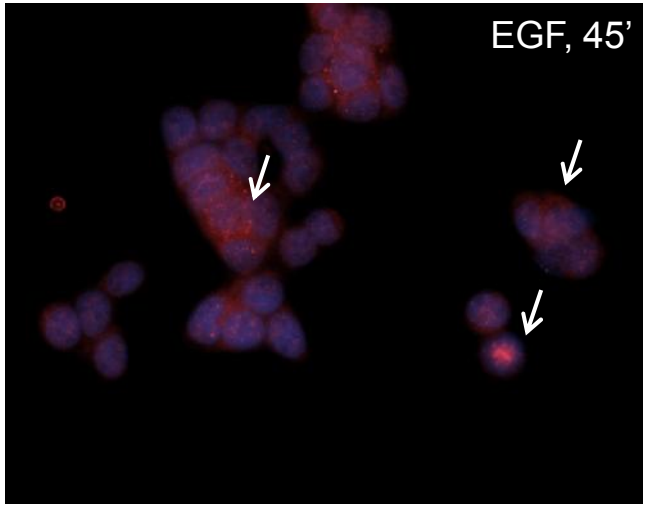


Figure 2

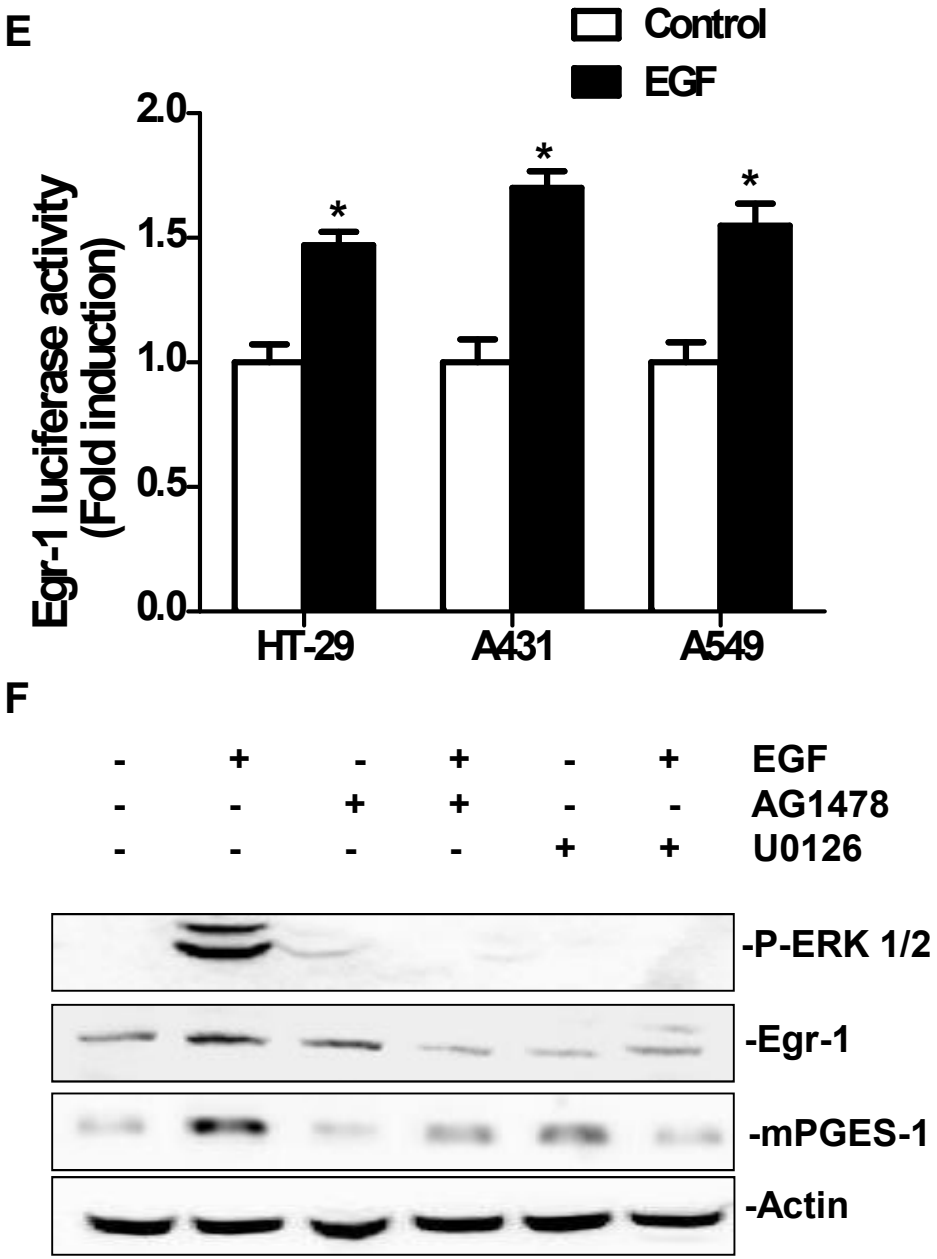
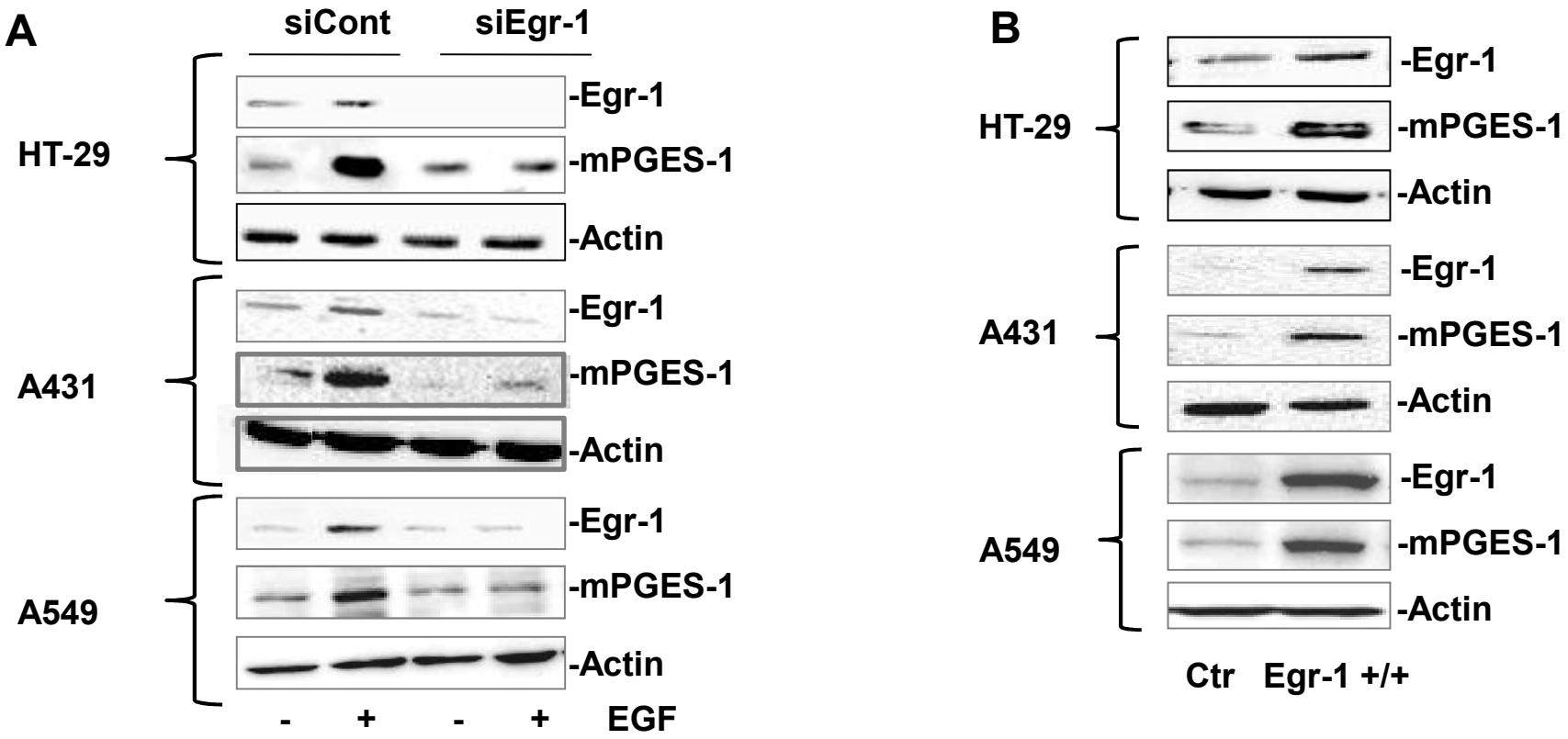
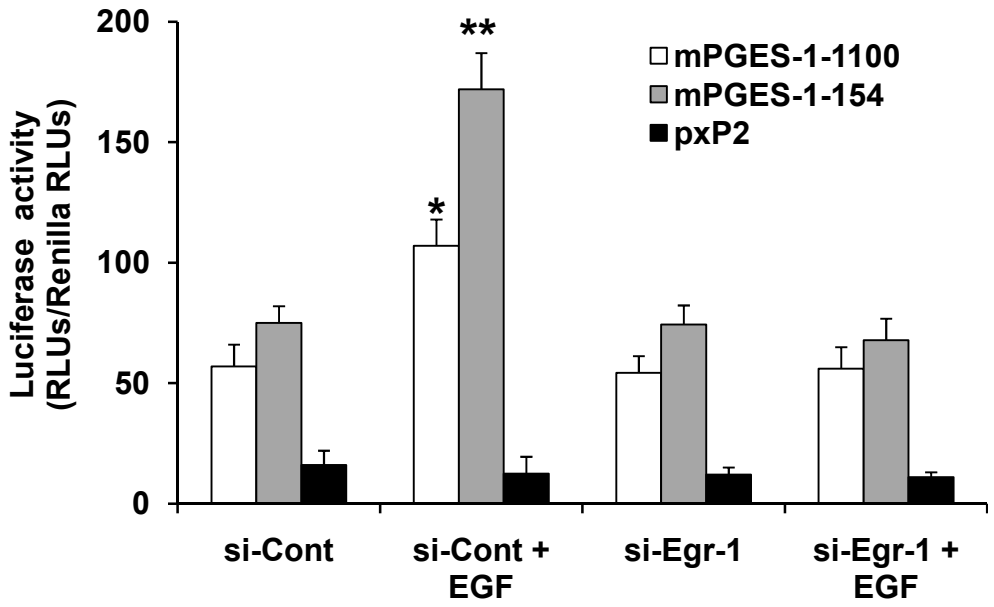

E
Control
EGF
2.0
1.5
1.0
0.5
0.0
Egr-1 luciferase activity
(Fold induction)
*
*
*
HT-29
A431
A549
F
- + - + - + EGF
- - + + - - AG1478
- - - - + + U0126
-P-ERK 1/2
-Egr-1
-mPGES-1
-Actin

Figure 3


A
siCont
siEgr-1
HT-29
-Egr-1
-mPGES-1
-Actin
A431
-Egr-1
-mPGES-1
-Actin
A549
-Egr-1
-mPGES-1
-Actin
- + - + EGF
B
HT-29
-Egr-1
-mPGES-1
-Actin
A431
-Egr-1
-mPGES-1
-Actin
A549
-Egr-1
-mPGES-1
-Actin
Ctr Egr-1 +/+

C


200
150
100
50
0
Luciferase activity
(RLUs/Renilla RLUs)
**
*
mPGES-1-1100
mPGES-1-154
pxP2
si-Cont
si-Cont +
EGF
si-Egr-1
si-Egr-1 +
EGF

D

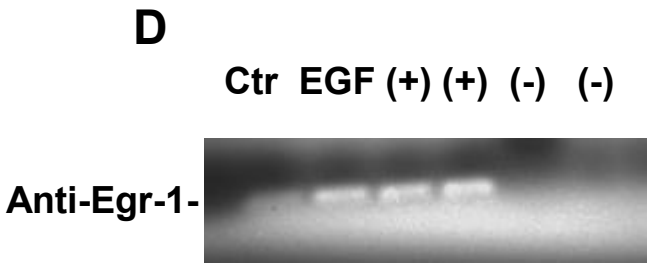
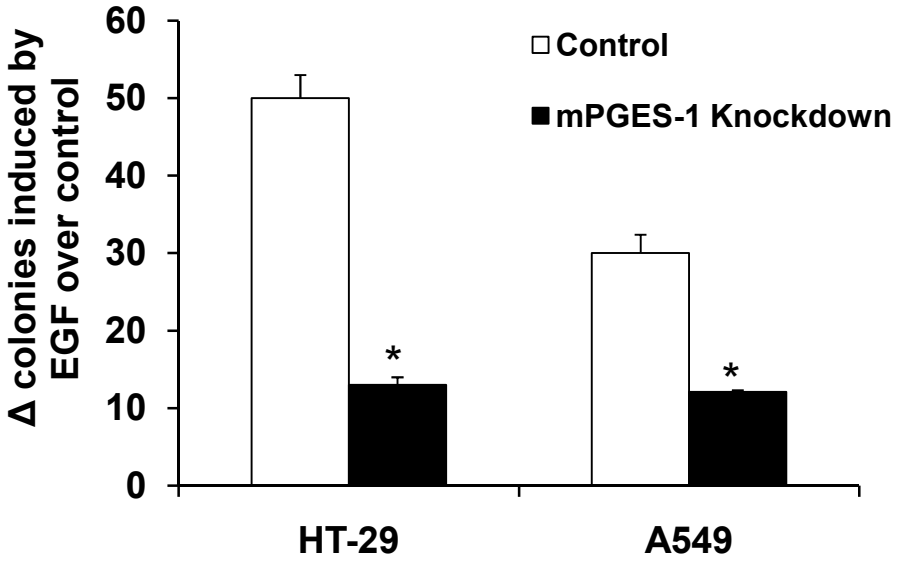

Ctr EGF (+) (+) (-) (-)
Anti-Egr-1-

Figure 4

A


Δ colonies induced by EGF over control
60
50
40
30
20
10
0
Control
mPGES-1 Knockdown
*
*
HT-29
A549

B

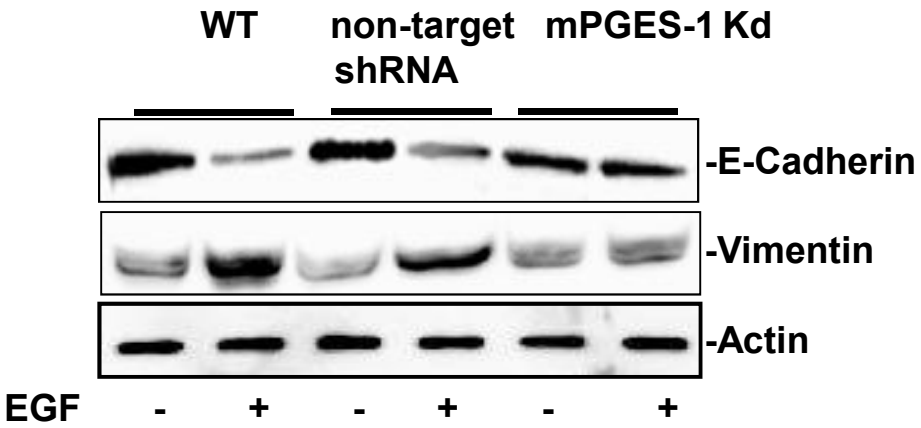
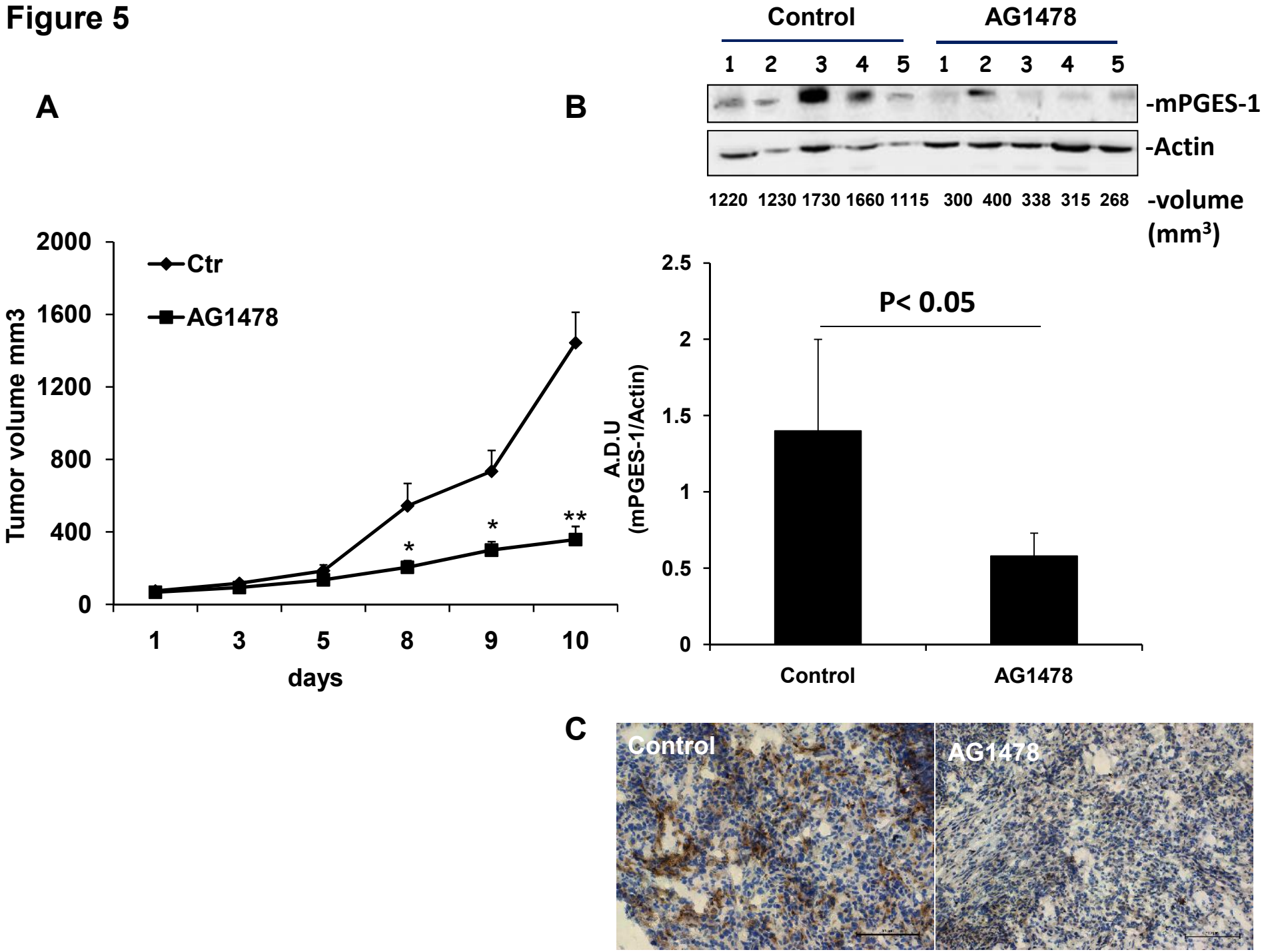

WT
non-target shRNA
mPGES-1 Kd
-E-Cadherin
-Vimentin
-Actin
EGF - + - + - +

Figure 5


A
B
C
Control
AG1478
1 2 3 4 5 1 2 3 4 5
-mPGES-1
-Actin
1220 1230 1730 1660 1115 300 400 338 315 268
-volume (mm3)
Tumor volume mm3
2000
1600
1200
800
400
0
Ctr
AG1478
*
*
**
1 3 5 8 9 10
days
A.D.U (mPGES-1/Actin)
2.5
2
1.5
1
0.5
0
P< 0.05
Control
AG1478
Control
AG1478